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MOLECULAR METHODS FOR DETECTING GUAR GUM ADDITIONS TO LOCUST BEAN GUM

5 Technical field

Locust bean gum, synonym of algaroba gum and carob bean gum, extracted from the seeds of carob trees (*Ceratonia siliqua*), and guar gum, synonym of gum cyamopsis and guar flour, extracted from the seeds of guar plant (*Cyamopsis tetragonolobus*) are used as food additives.

They are mainly used as thickening, emulsifying, stabilizing, or gelling agents in the food

10 industry. As described in the Official Journal of the European Communities from 9.12.98, pages L334/11 through L334/13, locust bean gum is coded as additive E 410 and guar gum has the code E 412, and these code numbers will be used in the present as synonyms of the above gums. As food additives, E 410 and E 412 can be used alone or together, or even

15 440i). The addition of these additives to foods provide general effects such as body, smoothness, and chewiness. Examples of these additions and of the effects they provide are: delaying the melting of ice creams, reduction of solids loss and speeding of curd formation in soft cheese manufacturing, and binding in composite meat products such as salami and sausages. Besides these food products, jams and jellies, milk products, breakfast
20 cereals, soups and soup mixes, sauces, gravies, toppings and syrups, and other food categories, may have additions of either or both E 410 and E 412 up to maximum usage levels permitted of between 0.15 % and 2.0 %, depending on the food category and on the additive.

Background art

As described in the Official Journal of the European Communities [European, C. 1998.

Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities 9.12.98:L334/11], E 410 and E 412 can be differentiated by microscopic

5 examination of their aqueous solutions stained with iodine and potassium iodide. In such preparations, E 410 presents long stretched tubiform cells, separated or slightly interspaced.

Their brown contents are much less regularly formed in guar gum than in locust bean gum. E 412 shows close groups of round to pear shaped cells. Their contents are yellow to brown.

Thus, theoretically, this microscopy methods should allow identification of guar in mixtures

10 of of guar gum and locust bean gum.

E 410 and E 412, individually or mixed, can be added as food additives, provided that the food label indicates whether only one of these two or a combination of the two additives is present. Both E 410 and E 412 are galactomannans and differences in their chemical

composition have been described: galatose:mannose ratios of 1:4 and 1:2 , in locust bean gum

15 and guar gum, respectively. Thus, isolated E 410 and E 412 can easily be differentiated from each other by gas-liquid chromatography of the hydrolyzed and derivatized polysaccharides,

and after calculation of the galactose:mannose ratio in the resulting chromatogram. However, for these two polysaccharides, and depending of their origin, supplier, and batch, variations in the degree of substitution of the mannan main chain have been described. For example, 37%

20 in E 412 and 23% in E 410 [**Cheetham, N. W. H., B. V. McCleary, G. Teng, F. Lum, and Maryanto.** 1986. Gel-permeation studies on xanthan-galactomannan interactions. Carbohydr.

Polym. 6:257-268.], galactose:mannose ratios for E 410 and E 412 of 1:2.69 and 1:1.44,

respectively [**Angelini, E., C. Boniglia, M. Mosca, and G. Bellomonte.** 1984. Detection and determination of plant gums by gas-chromatographic determination of their constituent

monosaccharides. *Rivista della Societa Italiana di Scienza dell'Alimentazione*. **13**:479-484.], or 3.01:1 and 1.48:1, respectively [**Preuss, A., and H. P. Thier.** 1982. Quantitative analysis of natural thickeners and gums by methanolysis and capillary column gas chromatography. *Z. Lebensm. Unters. Forsch.* **175**:93-100]. Even galactose:mannose ratios ranging from 3.1:1 to 7.7:1 were determined in locust bean gum fractions solubilized at temperatures between 20°C and 80°C [**Lopes da Silva, J. A., and M. P. Gonçalves.** 1990. Studies on a purification method for locust bean gum by precipitation with ethanol. *Food Hydrocolloids*. **4**:277-287.].

Due to these variations in composition it may be technically impossible in some mixtures of E 410 and E 412 to ascertain the individual content of each gum, and, for the same reasons, a possible adulteration of E 410 with E 412 may be technically very difficult to demonstrate. Cases of adulteration of E 410 with E 412 have been documented. The interest of this type of adulterations can be deduced from the different prizes of the two additives, with E 412 being cheaper than E 410.

Polysaccharides of plant origin are widely used as stabilizing agents in the food industry, and thus many chemical and some physical methods have been used to identify and quantitate these agents in mixtures and foods. None of these methods reviewed by Morley et al.

[**Morley, R. G., G. O. Phillips, and D. M. Power.** 1972. Fractionation and identification of commercial hydrocolloid stabilising agents. *Analyst*. **97**:315-319] neither the sequential fractionation scheme described in the cited reference can distinguish E 410 and E 412. More recently, the techniques generally used to identify and quantitate food additives of polysaccharide nature, the same general type of E 410 and E 412, including electrophoresis, gas chromatography and gas-liquid chromatography. None of these methods differentiate E 410 and E 412. Limitations of these methods, particularly when applied to the analysis of foods, are the need of complex extraction prior to analysis and expensive equipment.

Furthermore, hydrolysis, the first necessary step for chromatographic analysis of a mixture of E 410 and E 412, will also release, for example, mannose from xanthan, thus affecting the galactose:mannose ratio. Xanthan is another food additive (E 415) which is often used in combination with E 410 and E 412.

- 5 In a different described method, E 410, E 412, and other polysaccharides used as food thickeners were isolated, pyrolysed, and their products of pyrolysis were analyzed by gas chromatography. Although it was possible to identify a single food thickener by retention index monitoring and selected ion monitoring of the pyrolytic products, accurate analysis of a mixture of food thickeners was not achieved [Sjoeberg, A. M., and H. Pyysalo. 1985.
- 10 Identification of food thickeners by monitoring of their pyrolytic products. J. Chromatography. 319:90-98.].

- An assay using the *Bandeiraea simplicifolia* lectin which allows detection/quantification of guar and locust bean gum in commercial food products has been described [Patel, P. D., and G. B. Hawes. 1988. Estimation of food-grade galactomannans by enzyme-linked lectin assay.
- 15 Food Hydrocolloids. 2:107-118.]. Although the referred method detected E 410 and E 412 and not other food additives of polysaccharide nature (xanthan, carrageenan, alginates, and pectin), guar and locust bean gums could not be differentiated,.

- Finally, DNA-based methods have been described for the detection and/or identification of plants and plant-derived products. For example, in patent WO/9814607, a method is described
- 20 for detecting a particular plant species in foodstuffs. The method is based in the detection of plant-specific sequences located in the chloroplast DNA. Regions of the chloroplast DNA that contain characteristic sequences of some example plants were identified in the cited patent, allowing the differentiation between oranges and mandarines. Although the methods

described in the patent may be applicable to the differentiation of plant species other than those these described in the examples of this patent, these methods were not applied to the differentiation of either guar plant and carob tree neither to their derived products E 410 and E 412, respectively. On the other hand, there are reports of sometimes insufficient phylogenetic resolution in studies based on chloroplastic DNA, i.e., that they do not always allow species identification [**Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J. Donoghue.** 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Garden.* **82**:247-277.]. Additionally, as of today, there are not chloroplastic sequences available for the guar plant, and its differentiation from carob tree and other plant species based on these DNA sequences would require first their identification, isolation and sequencing. Finally, for the reasons detailed below, the extraction of DNA, either chloroplastic or not, from thickening agents, may require special methods that are not described in the cited patent.

For the present patent, it is finally relevant the description of a DNA based-method for the detection of wheat contamination in 35 samples of foods and food additives [**Allmann, M., U. Candrian, C. Höfelein, and J. Lüthy.** 1993. Polymerase chain reaction (PCR): a possible alternative to immunochemical methods assuring safety and quality of food. Detection of wheat contamination in non-wheat food products. *Z. Lebensm. Unters. Forsch.* **196**:248-251]. This method is based on the use of PCR and primers specific for the ribosomal DNA, i.e., on sequences of wheat DNA encoding for the RNA components of the ribosomes. It is important for the present patent to note that the authors of the cited method concluded that thickening agents or additives, in particular guar gum and locust bean gum, could not serve as a substrates for DNA isolation, and that the thickening and absorption effects of these additives made DNA extraction impossible.

Description

-Brief description of the invention

We describe the existence of diagnostic DNA sequences that allow identification of the plants from which guar and locust bean gums are extracted. These sequences have also been
 5 detected in the guar and locust bean gums, thus their detection in the DNA extracted from these gums ensure that their origins are either the guar plant or the carob tree.

Using PCR and the conserved primers (initiators) described in Figure 1A, we have amplified two DNA regions named ITS1 and ITS2 from the DNA extracted from the seeds of *Ceratonia siliqua* and *Cyamopsis tetragonolobus*, i.e., the plants from which the locust bean gum (E
 10 410) and guar gum (E 412) are extracted. Analysis of the amplification products with restriction endonucleases showed restriction patterns specific of each. Direct DNA sequencing and comparison of the aligned sequences showed that both plants can be differentiated by the presence of nucleotides specific of each plant species found in certain positions of the sequence. Furthermore, scrutiny for conserved and variable nucleotides within these
 15 sequences indicated the existence of two regions in the DNA amplified from the guar plant, that we name PG21 (SEQ ID NO5) and PG22 (SEQ ID NO 6), whose sequences can be used as primers for the specific amplification by PCR of a region of guar DNA within the ITS2 region. Sequences and positions of these primers specific of guar are shown in Figures 1A and 3. Once the specific primers PG21 and PG22 were tested for amplification of DNA extracted
 20 not from seeds but from commercial preparations of locust bean gum (E 410) and guar gum (E 412) we concluded that the usual methods described in the literature to extract DNA do not allow PCR amplification from these samples. Thus, we developed alternative methods for the extraction of DNA from these gums, which allowed DNA amplification. Using primers PG21 and PG22, we obtained amplification by PCR from the DNA extracted of all known samples

of guar gum, and no amplification from any of control samples of locust bean gum.

Furthermore, we demonstrate that with these DNA extraction and amplification methods we have detected guar DNA both in known control mixtures of guar and locust bean gum and in some commercial preparations labeled as "locust bean gum" which, theoretically, should not contain guar.

-Detailed description of the invention and examples.

As used herein, the term locust bean gum or E 410 is the ground endosperm of the seeds of the natural strains of carob tree *Ceratonia siliqua* [European, C. 1998. Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities.

9.12.98:L334/11]. The term guar gum or E 412, as used herein, is the ground endosperm of the seeds of natural strains of the guar plant [European, C. 1998. Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities.

9.12.98:L334/11]. For a detailed description of both terms we conform to what is described in the reference [European, C. 1998. Commission Directive 98/86/EC of 11 November 1998.

Official Journal of the European Communities. 9.12.98:L334/11]. Although this invention does not claim taxonomical issues, some of the sequences described here as part of the claims may have taxonomical applications. Thus, the taxonomical status of carob tree and guar plant species requires further explanation. For example, in reference [European, C. 1998.

Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European

Communities. 9.12.98:L334/11] both plant species are considered as belonging to the family *Leguminosae*, whereas other taxonomical sources, such as the National Center for

Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html>)

prefers to use the family name *Fabaceae*. We consider herein both family names as synonyms, in accordance with considerations of the International Code of Botanical Nomenclature

(Tokyo Code, Koeltz Scientific Books, Germany, 1994). Another taxonomical issue refers to the scientific name of the guar plant, cited as *Cyamopsis tetragonolobus* in reference

[European, C. 1998. Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities. 9.12.98:L334/11] or as *Cyamopsis tetragonoloba* by the NCBI

5 taxonomy database. Again, we will consider herein both species names as synonyms of the guar plant.

The terms DNA, PCR, primers, and sequences, are common for those skilled in the molecular biology field. We would refer to them herein as follows. DNA refers to deoxyribonucleic acid. PCR refers to methods for in vitro amplification of DNA where two synthetic

10 oligonucleotide primers, which are complementary to two regions of the target DNA (one for each strand) to be amplified, are added to the target DNA, in the presence of excess deoxynucleotides and a heat-stable polymerase (Taq polymerase). In a series (typically 30) of temperature cycles, the target DNA is repeatedly denatured, annealed to the primers, and a daughter strand extended from the primers. The daughter strands themselves (sometimes

15 referred as amplicons) act as templates for subsequent cycles, where DNA fragments matching both primers are amplified exponentially. Primer, or initiator, refer to a short pre-existing polynucleotide chain to which new deoxyribonucleotides can be added during the PCR process. Primers PG21 (SEQ ID NO 5) and PG22 (SEQ ID NO 6) have been produced synthetically, and are of sufficient length and with sequences that ensure that they recognize

20 and anneal to specific DNA sequences within the ITS2 region of the genome of the guar plant and they do not anneal to the carob tree genome. DNA sequences refer to the relative order of DNA bases or nucleotides (A, C, T, G), whether in a fragment of DNA, a gene, a chromosome, or an entire genome.

The regions known as "internal transcribed spacer" or ITS are constituents of the 18S-26S nuclear ribosomal DNA (rDNA), which encodes for the RNA component of the ribosomes (rRNA). The ITS are useful for phylogenetic studies of angiosperm plants [**Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J.**

- 5 **Donoghue.** 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Garden.* **82**:247-277]. The organization of these regions in the plant genomes is as shown in Figure 1A: two ITS regions named ITS1 and ITS2 are separated by a conserved small region named 5.8S and flanked by the 18S and 26S regions. Whereas ITS1 and ITS2 are highly variable in sequence within different families
10 and genera of plants, the 5.8S, 18S, and 26S regions are highly conserved. The small size of the ITS regions (less than 300 nucleotides) and the fact that they are flanked by conserved sequences, facilitate their amplification by PCR with conserved oligonucleotide primers designed against the conserved flanking regions. The ITS regions, as well as the other components of the rDNA, are multiple copies in the plant genomes and this facilitates
15 isolation, detection, amplification, cloning, and sequencing, even from old samples like herbarium material. Variations in length (number of nucleotides) between ITS regions of plants, although limited, have been described, and in some cases serve to differentiate between two close species. But most importantly for the present patent, differences in the sequence in the ITS regions usually allows differentiation between species of the same genus.
- 20 The present invention describes methods for the identification of guar gum, alone and in mixtures of guar gum and locust bean gum, by means of DNA amplification and its related analysis methods. For this purposes, we use rDNA sequences of the 18S, 5.8S, 26S, ITS1, and ITS2 regions described in reference [**Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J. Donoghue.** 1995. The ITS region of nuclear

ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Garden.* **82**:247-277.] and in Figure 1A. Since these regions were previously unknown for the guar plant and carob tree, the first experiments of this invention were addressed to determine the sequences of the ITS1 and ITS2 regions in the genomes of these two plant species.

5 For these purpose, we isolated DNAs from the seeds of the guar plant and carob tree, and amplified them by PCR using the primer pairs ITS5/ITS2 (SEQ ID NO 1/SEQ ID 4) and ITS3/ITS4 (SEQ ID NO 2/SEQ ID 3) described in [White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR
10 Protocols. A Guide to Methods and Applications. Academic Press, Inc., San Diego]. These pairs of primers amplify respectively the ITS1 and ITS2 and part of their adjacent regions (see Figure 1A). The amplification products were directly sequenced or detected by electrophoresis in agarose gels, purified, cloned and sequenced. The resulting sequences were aligned using a computer program and the ITS1 and ITS2 regions and their boundaries were
15 identified. Identification was performed by comparison of the sequences obtained with other known sequences of plant rDNA. The procedures and corresponding results are described in detail in the experiments shown in Example I.

Analysis of the sequences obtained from guar and carob tree seeds demonstrated that both amplicons containing ITS1 and ITS2 differ in their compositions and sequences, depending
20 on the plant species. Analysis of these sequences by restriction with endonucleases (restriction enzymes or restrictases) can be performed with computer programs, like MacVector (Oxford Molecular Group PLC), in order to predict the sizes of the fragments that could be generated with each endonuclease. We performed these analysis on the computer (data not shown) and selected some restrictases to illustrate the usefulness of this type of analysis as a method to

differentiate the DNA from both plant species. For these purpose, we first amplified separately the ITS1 and ITS2 containing regions from DNA extracted from the seeds of guar plant and carob tree, as detailed in Example I. Amplification products were purified and digested with several restrictases that produced differential restriction patterns for guar plant and carob tree. The results of this type of analysis, and the corresponding experimental details, are shown in Example II.

Differences in the DNA sequences obtained from the DNA extracted the seeds of guar and carob tree suggested that a method based on PCR and on these sequences could be designed for the specific detection of guar. The detailed study of the sequences from the two plants (shown in Figures 2 and 3) showed regions where the differences between the sequences of the two plants were most prominent. As a non-limiting example of this strategy, we selected the regions labeled as PG21 and PG22 in Figure 3, and used them as guar specific primers for PCR. In these two regions, the sequences of the two plants differ in many nucleotides, particularly in the 3' regions of the indicated primers. It is well known in the field of PCR and molecular biology that differences in the sequence of the 3' regions of PCR primers, rather than in the 5' end, increase the specificity of PCR primers. As detailed in Example III, DNA extracted from seeds of guar plant was amplified with primers PG21/PG22, but no amplification was obtained for DNA extracted from seeds of carob tree. These demonstrated the specificity of the primers designed by us.

As mentioned before in this patent, PCR amplification of DNA from plants and other polysaccharide-rich environments is not always an easy task, because polysaccharides are good inhibitors of most enzymes used in molecular biology, such as polymerases, restrictases, and ligases [Michaels, S. D., M. C. John, and R. M. Amasino. 1994. Removal of polysaccharides from plant DNA by ethanol precipitation. *Biotechniques*. 17:274-276]. This

is exactly the situation one will find if a DNA-based method has to be devised for the detection of DNA in commercial preparations of guar and locust bean gums, because, by definition [**European, C.** 1998. Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities. **9.12.98:L334/11.**], these samples must contain

5 at least 75% polysaccharide (galactomannan). As an important number of methods and even commercial kits have been developed to overcome the problems caused by polysaccharides in DNA extraction and amplification, we tried several of these methods. We first tried the CTAB method [**Doyle, J., and J. J. Doyle.** 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19:11-15.**] used with success in this invention

10 to extract DNA from seeds (see Example I). We also assayed an SDS-based method [**Milligan, B. G.** 1994. Plant DNA isolation., p. 59-98. *In* A. R. Hoelzel (ed.), *Molecular Genetic Analysis of Populations. A practical approach.* Oxford University Press, Oxford.], an ethanol-based method [**Michaels, S. D., M. C. John, and R. M. Amasino.** 1994. Removal of polysaccharides from plant DNA by ethanol precipitation. *Biotechniques.* **17:274-276.**], and

15 the commercial kits Nucleon PhytoPure Plant DNA extraction kit (Amersham Life Science) and DNeasy Plant kit (Qiagen). We did not obtain DNA amplification by PCR from commercial samples of guar gum, locust bean gum, or from mixtures of guar and locust bean gum (data not shown) with none of these methods. These negative results were reported before by authors who could not obtain amplifications from guar and locust bean gums,

20 whereas they obtained amplifications from other plant-derived materials and foods using the same experimental conditions [**Allmann, M., U. Candrian, C. Höfelein, and J. Lüthy.** 1993. Polymerase chain reaction (PCR): a possible alternative to immunochemical methods assuring safety and quality of food. Detection of wheat contamination in non-wheat food products. *Z. Lebensm. Unters. Forsch.* **196:248-251**]. These authors, when referring to the

25 negative results obtained for guar and locust bean gums, concluded that "...the thickening and

absorption effects made any extraction impossible..." of the DNA. Since none of the above mentioned methods produced positive results, we decided to investigate and develop our own methods for DNA extraction from locust bean and guar gums.

For these purpose, locust bean and guar gum (0.1-10%, w/w) and mixtures of these gums with known percent contents of each gum were suspended in either water ("water method"), buffers with different ionic strengths and compositions ("Tris method"), acetonitrile solutions of different compositions ("acetonitrile method"), or ethanol solutions ("ethanol method") at temperatures ranging from 4 °C to 65 °C. Suspensions were vortexed between 1 and 10 min and centrifuged. The supernatant fluids were recovered and used for PCR and for DNA and polysaccharide quantitations. In the ethanol method, the procedure described in the reference [Michaels, S. D., M. C. John, and R. M. Amasino. 1994. Removal of polysaccharides from plant DNA by ethanol precipitation. *Biotechniques*. 17:274-276] was followed, and at the end of the cited procedure, the dissolved material was used for the same PCR and quantitations used with the other extraction methods. For PCR amplification, the PG21/PG22 primers pair and the conditions described in Example III were used. The results obtained with these extraction methods are summarized in Table 1 and detailed in Example IV.

As explained in detail in example IV, although the water method extracts less DNA than the Tris method (the method that extracts more DNA for each gum or mixture assayed), the water method is the most adequate method of DNA extraction for its subsequent PCR amplification.

For these reasons, the water method was selected for future PCR experiments.

The usefulness of the methods described in this patent for the extraction and amplification of DNA from guar and locust bean gums and their mixtures was further verified by testing laboratory preparations of locust bean gum containing different known amount of guar gum, and by testing commercial preparations of locust bean gum. For these assays, the water

method of DNA extraction and the PCR method with the PG21/PG22 primers were used.

Analysis of the PCR products by direct electrophoresis or restriction analysis (see Example V for details) demonstrated that the methods described in this patent detect the presence of guar gum in mixtures of locust bean plus guar gum. It also demonstrates that among the

5 commercial preparations labeled as "locust bean gum" analyzed, some of them contained guar gum.

The methods described in this patent rely on DNA sequences that are specific (diagnostic) of the guar plant and carob tree. Given their specificity, these DNA-based methods ensure the compliance with the legislative requirements of E 410 and E 412 as galactomannans extracted
10 from the seeds of the species *Ceratonia siliqua* and *Cyamopsis tetragonolobus*, respectively [European, C. 1998. Commission Directive 98/86/EC of 11 November 1998. Official Journal of the European Communities. **9.12.98**:L334/11]. Consequently, an additional result of the methods described here is that they confirm the authenticity of the locust bean gum and guar gums received from suppliers.

15 **Brief description of the drawings**

Figure 1A shows the organization of the 18S-26S rDNA region. The relative positions of PCR primers used for the amplification of ITS1 and ITS2 containing regions, respectively the primer pairs ITS5/ITS2 and ITS3/ITS4, are shown. It also shows the position of the primers pair PG21/PG22 used for the specific amplification of an internal region of the ITS2-
20 containing region of guar. Sequences of primers ITS5, ITS2, ITS3, and ITS4 have been described in [White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR Protocols. A Guide to Methods and Applications. Academic Press, Inc., San Diego].

Figure 1B shows the results of the electrophoretic analysis in agarose gels of the PCR amplicons from DNA extracted from guar and carob tree seeds. Samples were amplified by PCR using the primer pairs ITS5/ITS2 (gel lanes 1 and 2) and ITS3/ITS4 gel (lanes 3 and 4) and therefore contain the ITS1 region and the ITS2 region, respectively. Carob tree amplicons are in lanes 1 and 3, and guar amplicons correspond to lanes 2 and 4. Lane St corresponds to DNA molecular mass markers, whose sizes (in nucleotides) are indicated on the left side of the figure. Samples were detected by electrophoresis on agarose gels, staining with SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes) and UV observation.

Figure 2 shows the sequences of the products shown on lanes 1 and 2 of Figure 1B, i.e., ITS1 containing regions of guar plant and carob tree. Products were sequenced using the ITS5 and ITS2 primers (boxed in the figure). Sequence names (identifiers) correspond to EMBL/GenBank/DDBJ DNA accession numbers. Sequence identifier AJ245575 (SEQ ID NO 8) corresponds to carob tree, and sequence identifier AJ245578 (SEQ ID NO 7) corresponds to guar. Sequences were aligned using the MacVector program, and dashes correspond to sequence gaps automatically introduced by the program to improve the alignment, whereas asterisks indicate nucleotide positions conserved in the two sequences. The ITS1 region (underlined) was determined by comparison to published sequences of several angiosperm plants [**Hershkovitz, M. A., and E. A. Zimmer.** 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Research*. **24**:2857-2876.].

Figure 3 shows the sequences of the products shown on lanes 3 and 4 of Figure 1B, i.e., ITS2 containing regions of guar plant and carob tree. Products were sequenced using the ITS3 and ITS4 primers (boxed in the figure). Sequence identifiers are accession numbers of the EMBL/GenBank/DDBJ DNA databases. Sequence identifier AJ245576 (SEQ ID NO 10) corresponds to carob tree, and sequence identifier AJ245577 (SEQ ID NO9) corresponds to

guar. Sequences were aligned using the MacVector program, and dashes correspond to sequence gaps, whereas asterisks indicate conserved nucleotide positions. The ITS2 region (underlined) was defined by comparison to published sequences of several angiosperm plants [Hershkovitz, M. A., and E. A. Zimmer. 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Research*. **24**:2857-2876.]. The sequences of primers PG21 and PG22 designed in this patent are also shown.

Figure 4 shows an example of a restriction analysis of the ITS1 containing region of DNA extracted and amplified from the seeds of guar plant and carob tree. The ITS1 containing region was amplified by PCR with the ITS5/ITS2 primers pair and digested with endonucleases *BcnI*, *ClaI*, and *HaeIII* (lanes 1 and 2, 3 and 4, and 5 and 6, respectively). Odd lanes correspond to carob tree and even lanes to guar. Restriction products were detected as in Figure 1B. Lane labeled St contains molecular mass markers, whose sizes (in nucleotides) are indicated on the figure left side.

Figure 5 shows an example of a restriction analysis of the ITS2 containing region of DNA extracted and amplified from the seeds of guar plant and carob tree. The ITS2 containing region was amplified by PCR with the ITS3/ITS4 primers pair and digested with endonucleases *SmaI* (lanes 1 and 2), *XhoI* (lanes 3 and 4), and *HaeIII* (lanes 5 and 6). Odd lanes correspond to carob tree and even lanes to guar. Restriction products were detected as in Figure 1B. Lane labeled St contains molecular mass markers, whose sizes (in nucleotides) are indicated on the left side of the figure.

Figure 6. Electrophoretic analysis of the PCR amplicons obtained with the PG21/PG22 primers pair from DNA extracted from the seeds of guar plant (lanes 1 to 3). DNA extracted from carob tree seeds was also tested for amplification using the same conditions and primers,

but no amplification was detected (lanes 4 to 7). Amplicons were detected as in Figure 1B. Lane St contains molecular mass markers, whose sizes (in nucleotides) are indicated on the left side.

Figure 7. Electrophoretic analysis of the PCR amplification products of DNA extracted from guar and locust bean gums and mixtures of both gums. DNA was extracted by the water method and amplified using the PG21/PG22 primers pair. Lanes 1 and 2 show the amplicons from negative and positive control samples, locust bean gum (lane 1) and guar gum (lane 2), respectively. Lanes 3, 4, and 5 show the amplicons obtained from locust bean gum samples containing 30%, 20%, and 10% guar gum, respectively. Lanes 6, 7, and 8 show the amplicons obtained from locust bean gum samples containing 12%, 6%, and 2% guar gum, respectively. The locust bean gum analyzed in lane 1 was used for making all the gum mixtures. Mixtures on lanes 3 through 5 were prepared with the guar gum sample shown on lane 2, and mixtures on lanes 6 through 8 were prepared with a different guar gum source.

Figure 8. Electrophoretic analysis of the amplicons obtained from different commercial samples and providers and labeled as "locust bean gum". DNA was extracted from these samples using the water method and amplified by PCR with the PG21/PG22 primers pair. Lanes contained amplicons from the commercial samples of locust bean gum (1 through 5), a negative control (locust bean gum, lane 6) and a positive control (guar gum, lane 7).

Figure 9. Restriction analysis of the amplification products shown in Figure 8 restricted with the *TaqI* (lanes 2 through 4) and *XhoI* (lanes 5 through 7) enzymes. Lanes 2 and 5, 3 and 6, and 4 and 7, contain respectively the amplification products of lanes 2, 5, and 7 of Figure 8. Lane 1 shows the unrestricted amplicon from Figure 8 lane 7 (control guar amplicon).

Example I.**Amplifications and sequences of the ITS1 and ITS2 of the DNA extracted from the seeds of guar plant and carob tree**

Seeds of guar plant and carob tree were mechanically scarified, embedded in sterile water
 5 overnight and germinated on moist filter paper in Petri dishes. Total DNA was extracted from
 20 mg of plant tissue using the CTAB protocol described in the reference [**Doyle, J., and J.
 J. Doyle.** 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue.
 Phytochem. Bull. **19**:11-15.]. The DNA content of each sample was quantitated in a
 fluorimeter using the PicoGreen^R dsDNA Quantitation Kit of Molecular Probes. Regions of
 10 the rDNA including the ITS1 and ITS2 were independently amplified by PCR using the
 primer pairs ITS5/ITS2 and ITS3/ITS4, respectively (sequences shown in Figure 1A and
 given in reference [**White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and
 direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H.
 Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR Protocols. A Guide to Methods and
 15 Applications. Academic Press, Inc., San Diego]). The parameters of the PCR reaction (35
 cycles) were 94 °C for 1.5 min, 56 °C for 1.5 min, and 72 °C for 1.5 min per cycle and a final
 extension cycle (10 min) at 72 °C. Reaction mixtures (final volume 0.05 ml) contained: 250
 µM of each dNTP, 0.5 µM of each primer, 1.25 units of Taq DNA polymerase, and 100 ng of
 DNA extracted from seeds, in 1 X Taq DNA polymerase reaction buffer supplied by
 20 Pharmacia. The amplification products were resolved by electrophoresis in low melting
 agarose gels and purified from them using the QIAquick Gel Extraction Kit (Qiagen) or
 Agarase (Roche). The agarose electrophoresis analyses were performed on 1 X TAE-3%
 agarose gels, and gels were stained with SYBR[®] Gold Nucleic Acid Gel Stain (Molecular
 Probes) and observed by UV illumination.

Figure 1B shows the electrophoretic analysis of the amplification products obtained from the DNA extracted from the seeds of carob tree (odd lanes) and guar plant (even lanes).

Amplicons shown on lanes 1 and 2 were obtained with the ITS5/ITS2 primers pair, thus

resulting in amplification of the rDNA regions containing ITS1. Amplicons shown on lanes 3

5 and 4 were obtained with the primers pair ITS3/ITS4, thus resulting in amplification of the rDNA regions containing ITS2.

The products visualized in Figure 1B were also sequenced on an Automated Applied

Biosystems 373 DNA Sequencer using the fluorescent dye terminator procedure and the

methods recommended by the supplier. Products were either directly sequenced or cloned in

10 the pGEM[®]-T vector (Promega) and sequenced (in such cases, at least 3 clones of each amplification product were analyzed to establish the sequence). For sequencing, the following primers shown in Figure 1A were used: ITS5 and ITS2, for sequencing the ITS1 products of Figure 1B (lanes 1 and 2), and primers ITS3 and ITS4 for sequencing the ITS2 products of Figure 1B (lanes 3 and 4).

15 Figures 2 and 3 show the resulting sequences aligned with the MacVector program. Sequence identifiers (sequence names) correspond to accession numbers given by the

EMBL/GenBank/DDBJ DNA databases. The boundaries of the ITS sequences were determined by comparison of our sequences with those of other angiosperm plants

[HersHKovitz, M. A., and E. A. Zimmer. 1996. Conservation patterns in angiosperm rDNA

20 ITS2 sequences. *Nucleic Acids Research*. **24**:2857-2876]. As shown in Figures 2 and 3 there are several nucleotide positions in the aligned sequences that are different between the guar

plant and carob tree sequences. Also, there are differences in the length of the sequences

obtained from the two plant species: the ITS1 containing region of the guar plant is 2

nucleotides longer than that of the carob tree, and the ITS2 containing region of the carob tree

sequence is 5 nucleotides longer than that of the guar plant. These differences in sequence length would suffice by themselves to distinguish if the amplification products originate from the guar plant or the carob tree, using methods that are known in the molecular biology field.

Example II.

5 Identification of guar and carob tree by amplification of DNA extracted from their seeds and restriction analysis of their ITS1 and ITS2 regions.

Amplifications of the ITS1 and ITS2 containing regions from the DNA of the guar plant and carob tree seeds were obtained as detailed in the previous example. These PCR products were purified from other reaction components with the High Pure™ PCR Product Purification Kit
10 (Boehringer). Purified amplicons were digested with the endonucleases detailed in Figures 4 and 5 using the conditions recommended by the endonucleases manufacturers (Pharmacia, Boehringer). The resulting restriction products were analyzed by electrophoresis in 1X TAE-4% agarose gels, and were detected and visualized as in previous electrophoretic analyses.

Figure 4 shows the results of the restriction analysis of the ITS1 containing regions amplified
15 from the seeds of carob tree (odd lanes) and guar plant (even lanes), restricted with endonucleases *BcnI* (lanes 1 and 2), *ClaI* (lanes 3 and 4), and *HaeIII* (lanes 5 and 6). Clearly, as shown in the figure, this type of analysis could differentiate the guar DNA of this region from the same region of the carob tree DNA. For example, *BcnI* did not cut the carob tree DNA whereas this enzyme generates two fragments of 247 and 98 nucleotides in the guar
20 sequence.

Figure 5 shows the same type of analysis explained in the last paragraph but for the ITS2 containing regions. This Figure shows that, for example, the *SmaI* enzyme generates two fragments of 298 and 112 nucleotides from the carob tree DNA (lane 1), whereas no restriction was seen for the guar DNA (lane 2).

The examples shown in Figures 4 and 5 are non-limiting: using the same methodology but with other restrictases that are commercially available it would be possible to produce differential restriction patterns from the DNA of the two plants under study. In this type of restriction analyses, a prediction of the usefulness of the different endonucleases can be performed by computer-assisted restriction analysis of the sequences, using MacVector or other computer programs.

Example III

Design and use of PCR primers specific of the DNA extracted from guar seeds

DNAs extracted from control seeds of guar and carob tree plants by the CTAB method (see example I) were amplified using primers PG21 and PG22 and the same PCR conditions detailed in example I, except that the annealing temperature was 55 °C. The amplification products were detected by electrophoresis on 1X TAE-3% agarose gels, and visualized by ethidium bromide or SYBR® Gold staining and UV observation.

Figure 6 shows the results of this type of experiments, and demonstrates that primers PG21 and PG22 amplify specifically the DNA extracted from guar and not from carob tree. The amplification fragment should have a length of exactly 194 nucleotides, as deduced from sequence AJ245577 (Figure 3) in the region comprised between PG21 and PG22. As shown in Figure 6, the fragment amplified from different guar seeds runs in the gels slightly below the 200 nucleotides marker, i.e., with the expected molecular mass.

Example IV

Extraction of DNA from guar and locust bean gums and their mixtures

DNA extraction from guar and locust bean gum and their mixtures was performed as follows. Ten mg of gums, obtained as powders from the suppliers, were suspended in 10 ml of either water (water method), 10 mM Tris-HCl pH 8.5 (Tris method), water: acetonitrile 7:3

(acetonitrile method), or 35% ethanol (ethanol method). Suspensions were vortexed for 5 min and centrifuged for 1 min at 15,000 x g. Supernatant fluids were recovered and used for PCR and quantitation assays, except in the ethanol method, where the procedure explained in the reference [**Michaels, S. D., M. C. John, and R. M. Amasino.** 1994. Removal of polysaccharides from plant DNA by ethanol precipitation. *Biotechniques*. **17**:274-276] was followed, and the final pellet obtained by this method was dissolved in 0.5 ml of water. For quantitation assays of the DNA extracted by the above four methods, 0.1 ml samples for DNA and the PicoGreen^R dsDNA Quantitation Kit of Molecular Probes were used. For total sugars quantitation, 0.01 ml of samples (extracts) and the phenol-sulfuric acid method [**Keleti, G., and W. H. Lederer.** 1974. *Handbook of Micromethods in the Biological Sciences*. Van Nostrand Reinhold, New York] were used. For PCR amplification, 0.005 ml of extracts were amplified with the PG21/PG22 primers pair and the conditions described in Example III. The results obtained with these extraction methods are summarized in Table 1.

15

Table 1
Specific detection of guar gum, alone and in guar/locust bean gum mixtures, by PCR amplification of the DNA extracted from the gums. Dependence of the detection on the method used for DNA extraction.

20

Gums	Extraction method	DNA ¹	Hexoses ²	DNA/polysaccharide	PCR ³
Guar	H ₂ O	46.87	0.639	1 / 14,000	+
30% Guar ⁴	H ₂ O	8.6	0.523	1 / 60,000	+

10% Guar ⁴	H ₂ O	10.8	0.541	1 / 50,000	+
Locust bean	H ₂ O	14.22	0.572	1 / 40,000	-
Guar	Tris	77.3	1.056	1 / 14,000	+
30% Guar ⁴	Tris	156.1	0.361	1 / 2,500	-
10% Guar ⁴	Tris	232.5	0.494	1 / 2,000	-
Locust bean	Tris	509.7	0.721	1 / 1,500	-
Guar	Acetonitrile	4.84	0.013	1 / 3,000	+
30% Guar ⁴	Acetonitrile	1.72	0.019	1 / 11,000	-
10% Guar ⁴	Acetonitrile	1.94	0.030	1 / 16,000	-
Locust bean	Acetonitrile	1.72	0.029	1 / 17,000	-
Guar	Ethanol	3.21	ND ⁵	NA ⁶	-
30% Guar ⁴	Ethanol	0.3	ND	NA	-
10% Guar ⁴	Ethanol	0.64	ND	NA	-
Locust bean	Ethanol	0.86	ND	NA	-

¹In ng/ml. Quantitated with the PicoGreen^R dsDNA Quantitation Kit of Molecular Probes. ²In mg of dissolved polysaccharide detected using the phenol-sulfuric method [Keleti, G., and W. H. Lederer. 1974. Handbook of Micromethods in the Biological Sciences. Van Nostrand Reinhold, New York.] per mg of gum. ³Amplification (+) or no amplification (-) by PCR using the PG21/PG22 primers pair and detection as in Figure 1B. ⁴Percent (w/w) guar in the locust bean gum-guar gum mixtures. ⁵Not detected. ⁶Not applicable.

As shown in Table 1, the four assayed methods had different efficiencies in extracting the DNA from guar gum and locust bean gum. Clearly, the Tris method was the most efficient one, followed by the water, acetonitrile, and the ethanol methods. These efficiencies in DNA extraction do not always correlate with the amount of solubilized polysaccharide, as can be deduced from the Table column showing the DNA to polysaccharide ratios obtained for each solubilization method. When extracting individual gums (100% guar or locust bean gum), it is clear that the Tris method is, compared to the other methods, much more efficient for the extraction of DNA from locust bean gum than from guar gum. This can be deduced from the ratios of DNA extracted by each method from each gum: for locust bean gum, the amount of DNA extracted by Tris divided by the amount of DNA extracted by water is 35.8, whereas the same ratio for guar gum is 1.6.

The above results help to understand the results found when DNA extraction methods were assayed on locust bean gum with known additions (30% or 10%, w/w) of guar gum, and the results obtained when the DNAs extracted from the gums and their mixtures were assayed by PCR. Clearly, although the water method extracts less DNA than the Tris method from each gum or mixture assayed, the water method is the most adequate for detecting guar DNA extracted from guar gum or guar/locust bean gum mixtures. This is demonstrated in column labeled as "PCR" in Table 1, where the results of PCR amplification with the guar specific primers PG21/PG22 are shown. Clearly, although the Tris method extracts more DNA than the water method from locust bean gum containing 30% or 10% guar gum, only the water method results in positive amplification and detection of the guar DNA contained in these mixtures. These extraction and PCR results suggest that most of the DNA extracted from gums mixtures using the Tris solvent should actually correspond to locust bean DNA, rather

than guar DNA, whereas with the water method sufficient guar DNA is extracted from guar/locust bean gum mixtures to allow its PCR amplification with the guar specific primers PG21/PG22.

In summary, the results obtained advised to use the water method for the detection of guar gum in mixtures of guar gum and locust bean gum by the PCR methods described in this patent.

Example V

Specific detection of guar DNA in guar gum and in mixtures of guar gum and locust bean gum

In this example, laboratory preparations of locust bean gum containing different known amounts of guar gum and commercial locust bean gum samples were tested. DNA extraction by the water method described in the previous example and PCR amplifications with the PG21/PG22 primers described in previous examples were used. Results of these studies are shown in Figures 7 and 8.

Figure 7 shows the results of testing laboratory mixtures of locust bean containing 30%, 20% and 10% of guar gum. These three mixtures were prepared from the same two samples of locust bean gum and guar gum. Reasoning that perhaps different gum manufactures may produce guar gums with different amounts of DNA, depending on their production processes or other reasons, we also prepared mixtures of the same locust bean gum plus a different guar gum. These locust bean gum mixtures contained 12%, 6% and 2% guar gum. As shown in Figure 7, all the locust bean gum mixtures containing guar gum produced by PCR with the PG21/PG22 primers an amplification band of the expected size, independently of the amount of guar present in the mixture. Control samples containing only locust bean gum or only guar gum gave the expected results: negative (no amplification by PCR) for the locust bean gum and positive (PCR amplification) for the guar gum. It is worth noting that, confirming our

expectations, different commercial samples of guar gum contain different amounts of DNA. This can be deduced from the comparison of the results obtained, for example, from mixtures containing 2% and 10% guar gum (Figure 7 lanes 8 and 5, respectively). These two samples were prepared with the same locust bean gum but with guar gums from two different providers.

The usefulness of the methods described here for detecting guar gum was also tested on commercial preparations labeled as "locust bean gum". We obtained these commercial samples from different providers and selected some of them for study following the methods described in this patent. Particularly, we selected those samples producing viscosities higher than 250 cps at room temperature, since locust bean gum at this temperature usually produces viscosities not higher than 100 cps, and higher viscosities could be due to oversight or undeclared presence of guar gum. As shown in Figure 8, four out of the four theoretically pure locust bean gum preparations studied produced positive amplifications by PCR with the guar -specific primers pair PG21/PG22 (lanes 2 through 5 in this Figure). This indicates that the four mentioned samples contained guar, although in different proportions, as can be deduced from the different intensities of the gel bands (compare for example lanes 2 and 3). Control gums produced the expected results: locust bean gum produced no amplification (lane 6), whereas guar gum amplified (lane 7). One commercial sample labeled as "locust bean gum" with the usual viscosity values of locust bean gum was also negative by PCR (lane 1).

An additional proof of the guar presence in some commercial preparations labeled as "locust bean gum" was obtained by restriction analysis of the amplification products mentioned in the last paragraph. For designing this analysis, we followed the directions and methods used in Example II and the AJ245577 sequence shown in Figure 3. As shown in Figure 9, the amplification products seen in Figure 8 contained two restriction sites for endonuclease *TaqI*, which generates three fragments of 106, 52, and 36 nucleotides. The same enzyme would

generate (as shown by computer-assisted restriction analysis) only two fragments of 143 and 52 nucleotides on the equivalent carob tree sequence, i.e., the sequence comprised between the PG21 and PG22 primers. Additionally, the *XhoI* enzyme, which does not cut the carob tree sequence, produces two fragments in the amplicons, as expected from the guar sequence comprised between PG21 and PG22 sequences. Thus, the enzymes used in this example are diagnostic, but also other enzymes (as discussed in Example II) can be used for the same purpose.

Claims

1. Methods for differentiating the seeds of the plant species *Cyamopsis tetragonolobus* (or *Cyamopsis tetragonoloba*) and *Ceratonia siliqua*, said methods based on confirming the differences in the rDNA extracted from the seeds of these two species following these steps:

- i) the seeds are germinated and DNA is extracted from them,
- ii) the extracted DNA are amplified by PCR with primers ITS2, ITS3, ITS4, and ITS5 that are specific of the rDNA, and that are used in combinations as ITS5/ITS2 or ITS3/ITS4 pairs, and
- iii) the rDNA amplification products are detected

2. Methods for detecting the differences in the rDNA amplified from the seeds using the methods described on claim 1, said methods comprising sequencing of the rDNA products of amplification and comparison with the guar plant and carob tree rDNA sequences described in this patent. The degree of identity of the sequence obtained from an unknown seed with those of the guar plant or carob tree allows deduction of its correspondence to one of these two species. The sequences of carob tree are AJ245575 and AJ245576, and those of the guar plant are AJ245577 and AJ245578.

3. Methods for detecting the differences in the rDNA amplified from seeds using the methods described on claim 1, said methods not requiring DNA sequencing and that, taking advantage of the differences in the sequences described on claim 2, identify the amplified rDNA as arising from carob tree or guar. In these methods, the rDNA products
5 of amplification are:

- i) digested with restriction endonucleases that produce different fragments according to the sequence being digested, such as, but not exclusively, the enzymes *BcnI*, *ClaI*, *HaeIII*, *XhoI* or *SmaI*,
- ii) the digestion products are resolved by, for example, electrophoresis in agarose
10 gels and visualized by staining with SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes), ethidium bromide or other DNA detection reagents, and
- iii) the products of the digestion resolved in the gels are compared to those obtained from control guar and carob tree, so that the coincidence with the restriction patterns of the rDNA amplified from one or the other plant species allows the
15 attribution of the rDNA amplified and digested from an unknown seed to one of the two cited plant species.

4. Methods for the specific detection of the guar seeds based on the rDNA sequences of guar and carob tree described in claim 2, said method comprising the steps:

- i) these sequences are compared by alignment, and primers directed against the
20 guar sequences are designed. These primers are identical or similar to sequences of the guar plant but are different in the carob tree sequence, such as the PG21 and PG22 sequences described in this patent.
- ii) said oligonucleotides are used as primers for the amplification by PCR of the DNAs extracted from the seeds of guar, carob tree or from unknown seeds
25 suspected to belong to one of these two species, and

iii) the DNA products of amplification are detected by any of the methods described on claims 2 and 3.

5. Methods for detecting guar gum (E 412) alone or mixed with locust bean gum (E 410), said methods comprising DNA extraction from these gums or their mixtures, amplification by PCR and detection of the amplification products corresponding to guar.

6. Methods for the extraction of DNA from guar gum (E 412) and locust bean gum (E 410) and their mixtures, comprising the steps of:

i) a certain amount of these gums or their mixtures is weighed and suspended in a certain volume of, preferably, water, or a buffered or unbuffered aqueous solution, containing or not other components like acetonitrile or ethanol,

ii) the suspension is shaken for a variable time, preferably 5 minutes, at a temperature between 0 °C and 100 °C, preferably at room temperature,

iii) the solubilized materials are separated by decantation or preferably by centrifugation, for example at 15,000 x g for 1 minute, and

iv) a sample of the soluble material, i.e., the upper phase of the decantation or centrifugation step, is recovered for its amplification by PCR.

7. Methods for the amplification by PCR and detection of the amplification products of the DNAs extracted from guar gum, locust bean gum, and mixtures of these gums, following the methods of claim 6 and characterized by the use of the methods described on claims 2, 3, and 4.